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Detection of Gluten in Human Sera by an Enzyme Immunoassay: Comparison of Dermatitis Herpetiformis and Celiac Disease Patients with Normal Controls

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We have developed a triple sandwich enzyme immunoassay to detect circulating gluten in human sera. With human sera containing known amounts of added gluten as controls, the assay was sensitive in the range of 0.75 to 75 μg of gluten per ml of serum. Forty-one control subjects were compared to 21 patients with dermatitis herpetiformis and 11 patients with celiac disease. The dermatitis herpetiformis and celiac disease patients had significant elevation of serum gluten values over the control subjects. Circulating gluten antigenemia is a previously unrecognized feature which may be important in understanding the pathogenesis of dermatitis herpetiformis and celiac disease.

The association between dietary wheat protein ingestion and dermatitis herpetiformis (DH) has been observed since 1968 [1]. The wheat protein antigens felt to be responsible are gluten and particularly one subcomponent of gluten, called gliadin, which is soluble in dilute aqueous alcohol [2,3]. Gluten and gliadin have been shown to react equally with antibodies raised to either wheat protein [4]. Previous studies have shown the presence of antibodies to wheat proteins in sera of patients with DH [4–7]. Recently, Unsworth has demonstrated that the wheat protein gliadin binds to the reticulin-like fibers in the dermis of both normal and DH skin [8]. It has been suggested that wheat proteins bound to antibodies may circulate and bind to skin [9]. We report the detection of wheat protein in the sera of patients with dermatitis herpetiformis or with celiac disease (CD) by an enzyme immunoassay (EIA).

PATIENTS AND METHODS

Patients

Serum was collected from 73 subjects without regard to dietary wheat protein intake. Control sera was evaluated from 41 healthy adult volunteers (mean age 30 yr) with no skin or bowel disease. The control sera was compared to the sera of 21 patients with DH (mean age 45 yr) and 11 adults or children with CD (mean age 10 yr). The DH patients all had positive immunofluorescent biopsies of skin for granular IgA deposits in the dermal papilla. The CD patients all had bowel disease related to wheat protein ingestion and villous atrophy documented by small bowel biopsy.

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Abbreviations:
CD: celiac disease
DH: dermatitis herpetiformis
EIA: enzyme immunoassay
OD: optical density

Enzyme Immunoassay to Detect Wheat Protein in Sera

A triple antibody sandwich EIA was developed to detect wheat protein in sera. This technique is detailed in Table I. Antibodies to gluten were generated in two animal species, goat and rabbit, through bi-monthly immunization with a commercial wheat protein gliadin (ICN Pharmaceuticals, Inc., Cleveland, OH) in complete Freunds adjuvant. The sera from 3 different rabbits and 1 goat immunized to gliadin were evaluated by Ouchterlony double diffusion analysis [10]. Each sera was placed in wells adjacent to gluten and gliadin antigens. An immunoprecipitation line of identity for gluten and gliadin was seen in each serum evaluated. Goat serum was diluted in borate buffer for use in the enzyme immunoassay. The rabbit IgG was separated from the serum by DEAE ion exchange chromatography. The eluate was concentrated to the original serum volume over a UM 10 (Amicon, Lexington, MA) membrane. The antibody titer of the animal serum and immunoglobulin against gluten was evaluated by EIA [4] and was found to be greater than 1:10,000 (to be published).

Flat-bottomed polystyrene plates (Linbro Flow Labs, Hamden, CT) containing 96 wells were used for the assay as described in Table I and Fig 1. Controls for the assay were as follows: commercial grade wheat protein gluten (Sigma, St. Louis, MO) which contains gliadin and glutenin was mixed in 0.05 M borate buffered saline, pH 8.0, for 1 hr and then incubated at 4° C for 18 hr. The solution was then centrifuged at 2,200 g for 10 min and the supernatant separated. The supernatant was then filtered into a sterile container through a 0.2 μ (Nalge, Rochester, NY) filter. A dye binding protein analysis (Bio Rad, Richmond, CA) was done on the supernatant [11]. The measured protein content was considered to be the gluten content of the solution. The gluten solution was further diluted in 0.05 M borate buffered saline, pH 8.0, to known concentrations of 750, 75 and 7.5 μg of gluten per ml of buffer solution. To 900 μl of normal human serum, 100 μl aliquots of gluten in borate buffer or borate buffer was added, giving human serum controls with added gluten concentrations of 75, 7.5 and 0.75 μg of gluten per ml of serum. Nine hundred μl of sera diluted with 100 μl of borate buffered saline served as negative controls.

A horseradish peroxidase conjugated immunoglobulin fraction of rabbit anti-sera against goat immunoglobulin (Miles-Yeda, Ltd., Israel) was used to detect binding of goat antibodies to gluten in the wells (Table I). The color indicator substrate for horseradish peroxidase was prepared by the combination of 3 solutions. Twenty-five mg of 4-aminoantipyrine and 810 mg of phenol were dissolved in distilled water to give a total volume of 50 ml. One ml of a solution containing 1.0 ml of 3 % hydrogen peroxide in 10 ml distilled water was added to 50 ml of 0.2 M potassium phosphate buffer, pH 7.0. The final reagent was a combination of 42 ml of the phenol and 4-aminoantipyrine solution added to 45 ml of the hydrogen peroxide potassium phosphate buffer solution.

The optical density (OD) at 492 nm of each well was read by an 8-channel photometer (Titertek Multiskan Flow Labs, Hamden, CT) which recorded the OD values within 60 seconds. The OD was read on a scale from 0 to 2. The OD was blanked on an empty polystyrene plate.

The optimal concentrations of the various reagents was determined by checkerboard titration. The assay was first developed using known concentrations of gluten in 0.05 M borate buffered saline solution and then modified by the use of gluten added to human sera. The gluten was dissolved in the borate buffered saline because of the buffer's bacteriostatic properties [12] and the convenience of using a single buffer in the assay.

All serum samples, including controls with known amounts of gluten, were placed in quadruplicate wells. The mean OD value of the quadruplicate samples was calculated. For each polystyrene plate the mean

TABLE I. *Details of EIA to detect wheat protein in sera*

1. 100 μl of a solution containing 0.1% glutaraldehyde in 0.1 M carbonate buffer, pH 9.6, was added to each well of a 96 well Linbro polystyrene flat-bottomed plate and the plate was incubated at 37°C for 2 hr.
2. Three times each well was evacuated and then washed with approximately 300 μl of distilled water. After the final wash, each well was gently aspirated dry.
3. 100 μl of rabbit anti-gluten immunoglobulin diluted 1:200 in 0.1 M carbonate buffer, pH 9.6, was added to each well and the plate was incubated for 18 hr at 4°C.
4. Wells were emptied and washed 3 times as in step 2, however 0.05 M borate buffered saline, pH 8.0, with 0.05% Tween 20 was used in place of distilled water.
5. Sera to be tested was diluted 1:25 in 0.05 M borate buffered saline, pH 8.0, with 0.05% Tween 20. 100 μl of the diluted sera was added to each well. The plate was incubated at 37°C for 90 min.
6. Wells were emptied and washed 3 times as in step 4.
7. 100 μl of goat anti-gluten sera, diluted 1:100 in 0.05 M borate buffered saline, pH 8.0, with 0.05% Tween 20 was added to each well. The plate was incubated 90 min at 37°C.
8. Wells were emptied and washed 3 times as in step 4.
9. 100 μl of horseradish peroxidase conjugated rabbit immunoglobulin specific against goat immunoglobulin was diluted 1:200 in 0.05 M borate buffered saline, pH 8.0, with 0.05% Tween 20 and added to each well. The plate was incubated 60 min at 37°C.
10. Wells were emptied and washed 3 times as in step 4.
11. 100 μl of color substrate was added to each well.
12. After 1 hr incubation at 37°C the OD in each well was read and recorded by an 8 channel photometer.

OD values of the control samples with known amounts of added gluten were plotted against the log of the gluten concentration (Fig 2). The curve generated was used to calculate serum gluten concentrations for sera tested on the same plate. The correlation coefficient of the generated curve for gluten concentration was greater than .95 for all plates tested. By repeat testing, the assay appeared to be sensitive in the range of 0.75 to 75 μg of gluten per ml of serum. For the purposes of statistical comparison among groups, calculated values outside of this range were used. Fisher's Exact Test and the 2-tailed *t*-test were used to compare the various groups. Rabbit and goat sera, prior to immunization, contained no specific antigluten activity and were used as negative controls for the assay.

Two human sera with 4 known concentrations of gluten added were compared on the same polystyrene plate on 3 separate occasions. The correlation coefficient of the generated curve for gluten concentration was greater than 0.96 for each sera. The serum available in largest quantity was used to generate the standard curve for all subsequent assays. The percent error OD value, defined as the standard error of the mean OD value divided by the mean OD value, was calculated for the quadruplicate samples on the same plate. The average value of the percent error for all quadruplicate samples was 2.3%. The day-to-day variability of the assay was evaluated by repeatedly testing one control and one positive sera on different plates on 6 different days. The percent error of the control sera and positive sera were 3% and 2%, respectively. The positive control sera gave a greater value than the negative control sera on each day. The mean difference OD value between the 2 sera on 6 different days was evaluated with a 3.9% day-to-day variation.

RESULTS

Figure 3 shows the calculated gluten values for the 73 sera. By a 2-tailed *t*-test, both DH and CD patients, as a group, showed significant elevation of mean serum gluten values.

The data were evaluated for the number of subjects with calculated serum gluten values greater than the mean of the controls (0.278 μg/ml) plus 2 SD (SD = 0.27 μg/ml) (Table II). This value, 0.812 μg of gluten per ml of serum, is within the range of sensitivity of the assay. Comparing the DH and CD patients against the control subjects shows a highly significant difference by Fisher's Exact Test.

DISCUSSION

A relationship of immunologically mediated human disease to dietary antigens has often been postulated but rarely has it been shown that such antigens may enter the circulation relatively intact antigenically and may be available to elicit immunological reactions in organ systems outside the gastrointestinal tract. Wheat protein, especially the relatively insoluble protein complex, called gluten, and its subcomponent soluble in dilute alcohol, called gliadin, have been shown to be the dietary antigens responsible for precipitation of celiac disease, or nontropical sprue [2,3]. The appreciation that a skin disease, dermatitis herpetiformis, is associated with CD and may also represent a disease related to dietary wheat protein, led to speculation that wheat protein antigens may be absorbed and lead to disease at sites removed from the gastrointestinal tract [9,13]. The reports of circulating immune complexes in these diseases [14–16] and depression of serum complement after wheat protein ingestion [17] imply absorption and circulation of antigenic substances derived from wheat protein, but antigenemia has not been previously demonstrated in these conditions.

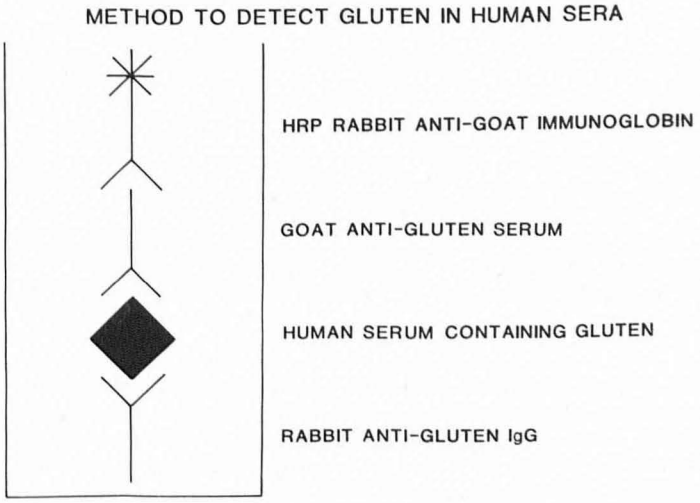

FIG 1. Schematic diagram of EIA to detect wheat protein in sera.

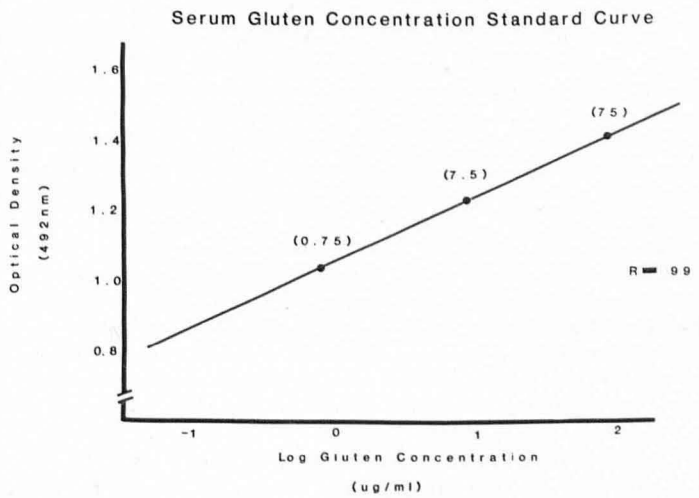

FIG 2. Sample standard curve used to calculate serum gluten concentration. Values in parentheses are those of the control samples with known amounts of added gluten.

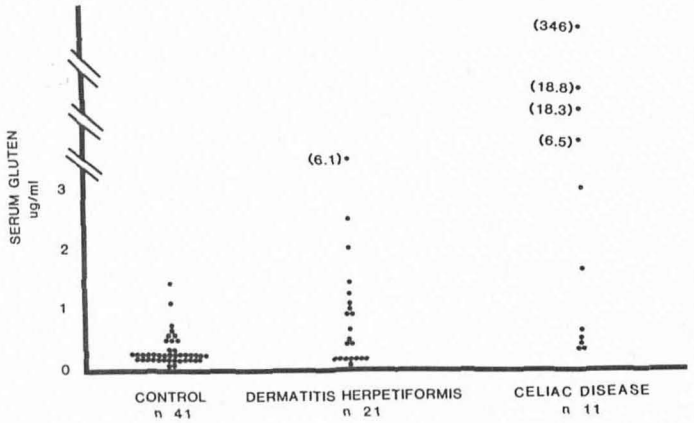

FIG 3. Calculated serum gluten concentration for 73 sera. Mean serum gluten values ± standard error of the mean for each group (μg/ml) was 0.278 ± 0.042 control), 0.943 ± 0.295 (DH) and 36.05 ± 31.12 (CD). The DH group ($p < 0.01$) and the CD group ($p < 0.05$) were significantly different from the control group by the 2-tailed *t*-test. Serum gluten values < 0.750 μg/ml were derived by calculations from the standard curve to allow statistical comparison among the groups. Individual sera containing < 0.750 μg/ml of gluten, may, in fact, contain no gluten since these values are less than the lower limit of sensitivity of the assay.

TABLE II. *Number of subjects with elevated serum gluten*

	n	No. positive by EIA[a]	p[b]
Control	41	2	
DH	21	8	0.003
CD	11	6	0.001

[a] Number positive by EIA indicate subjects with calculated serum gluten values greater than the mean of the controls plus 2 SD.
[b] *p*-values were derived from a 2-tailed Fisher's Exact Test.

The purposes of this study were to develop a sensitive assay for wheat protein antigens and to study the sera of patients with CD and DH for the presence of these antigens. We adapted an enzyme immunoassay for this purpose. With the use of the 96 well microtiter plate and an 8-channel photometer for rapid reading and recording of optical density values, this assay allowed easy study of many test sera in quadruplicate, inclusion of positive controls on each plate for generation of a standard curve, and study of many sera from normal control subjects. Because of the many layers in this sandwich technique (Fig 1), the optical density values generated in most wells were relatively high. Despite the high background, the precision of the assay was quite good, as demonstrated by the high correlation coefficients of the positive controls and the excellent agreement among the four wells used for specimens. The wheat protein antigen preparation used to generate the positive controls was crude, therefore, the true amounts of antigenic material in test sera must be less than the values calculated from our standard curve.

The results of this study show clearly that the sera of patients with DH and CD, compared to those of normal control subjects, contain antigenic material related to wheat protein. Both mean serum values and the number of sera with elevated values were significantly higher in these 2 diseases. If sera had been obtained after dietary challenge with wheat protein rather than in a random manner, these differences might have been further magnified.

It is possible that the higher levels of gluten found in CD and DH patients, in this study, reflect a simple diffusion of gluten across a damaged intestinal mucosa. As the enteropathy in CD is more severe than in DH, this would account for findings of higher serum gluten levels observed in CD patients. If wheat protein antigens circulate in both patients with CD and those with DH, other differences must exist to explain the presence of skin disease in DH and the absence of skin disease in CD patients.

CD is a chronic, symptomatic bowel disease characterized by diarrhea and malabsorption, whereas DH is a chronic, pruritic vesiculo-bullous skin disease associated with a largely asymptomatic bowel disease histologically similar to CD. Skin disease in DH patients may improve on a wheat protein-free diet, but the time required for response of the skin disease is far longer than for the bowel disease [18–21]. The role of wheat protein, if any, in the pathogenesis of the skin disease must involve other factors such as the differential immune response to wheat protein or skin changes allowing the interaction of circulating wheat proteins with skin. It would be of interest to know whether wheat proteins in serum change with short-term and long-term wheat protein-free diets in CD and DH patients and whether clinical improvement of skin disease correlated with decreases in serum wheat protein levels.

Knowledge that wheat protein antigenemia may occur in patients with DH is a fact that may allow the following previously known clues concerning the pathogenesis of this disease to be tied together: granular IgA deposits at the dermal-epidermal junction in the skin [22] presence of serum antibodies to wheat protein antigens in the serum [4–7], gluten sensitivity [1], and the binding of gliadin to reticulin fibers in the skin at the same site as the IgA deposits [8]. When wheat protein is absorbed and circulates, it may bind to the skin, fix IgA antibody at that site, and thereby lead to a wheat protein-induced skin disease. Further support of this hypothesis will require demonstration of specific IgA antibodies to wheat protein in these patients and the demonstration of wheat protein antigen and specific antibody in the skin.

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The Effect of Topical Drugs on Mouse Ear Epidermal Transglutaminase Activity

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The response of mouse ear epidermal transglutaminase to single applications of anthralin, retinoic acid (both 50 μg/ear) or fluocinolone acetonide (2 μg/ear) was determined. Anthralin and retinoic acid caused inflammation and accumulation of epidermal protein and DNA, whereas fluocinolone acetonide resulted in ear thinning and decreased epidermal protein and DNA. Treatment with either anthralin or retinoic acid caused increases in absolute amounts of epidermal transglutaminase activity/ear. Anthralin increased this parameter 70–100% above acetone-treated controls from 48 hr through 7 days. Retinoic acid-treated ears showed a slower initial increase but peaked at 4 times control level by 96 hr before returning to normal at 7 days. Fluocinolone acetonide treatment had no effect on this parameter. The specific activity of epidermal transglutaminase (total epidermal transglutaminase/total soluble epidermal protein) was decreased by retinoic acid treatment; was maintained at normal levels by anthralin (except for the 7-day point where it decreased 50%); and was dramatically stimulated by fluocinolone acetonide. In the latter case, specific activity was more than 5 × control by 96 hr and still near this level at 7 days. Epidermal transglutaminase activity is a marker of differentiation, and protein and DNA accumulation an indication of growth. Thus, at the doses studied, retinoic acid favors growth over differentiation, anthralin maintains a normal to near normal ratio of growth to differentiation, and fluocinolone acetonide strongly favors differentiation over growth.

Epidermal transglutaminase (ETG) a marker enzyme of terminal epidermal differentiation, is responsible for stabilization of the cornified envelope via formation of isopeptide bonds. Other than the report of Buxman and Wuepper [1] that histochemically this enzyme appears to be absent in psoriatic lesions lacking a granular layer, little is known of its activity during either the pathogenesis or resolution of epidermal diseases. Whereas the effect of topical drugs on macromolecules and enzymes associated with epidermal proliferation has been studied in great detail, relatively little is known concerning their effect on epidermal differentiation. Histological evidence has long indicated that a restoration of normal epidermal differentiation actually precedes the normalization of epidermal growth in psoriatic plaques [2,3]. Furthermore, acne, although associated with follicular hyperplasia, is clearly a disease of aberrant keratinization. We therefore feel it is important to understand the effect of known anti-psoriasis and anti-acne agents on epidermal differentiation of normal and diseased skin. In this initial investigation, we present the effects of single applications of anthralin, fluocinolone acetonide (FA) and retinoic acid (RA) on normal mouse ear epidermal transglutaminase. In addition, to interpret our results over the background of biological activity of these agents, changes in ear thickness and epidermal DNA and protein have been monitored.

MATERIALS AND METHODS

Mice

Female Swiss/Webster mice 5–6 weeks old obtained from Simonsen Labs, Inc., Gilroy, CA, were kept under conventional conditions and used after 2 weeks acclimation.

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Abbreviations:
ETG: epidermal transglutaminase
FA: fluocinolone acetonide
RA: retinoic acid